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<b>(21) International Application Number:</b> PCT/US98/08723 <b>(22) International Filing Date:</b> 30 April 1998 (30.04.98) <b>(30) Priority Data:</b> 08/852,394      7 May 1997 (07.05.97)      US <b>(71) Applicant:</b> TRUSTEES OF TUFTS COLLEGE [US/US]; Ballou Hall, 4th floor, Medford, MA 02155 (US). <b>(72) Inventors:</b> HUBER, Brigitte, T.; 175 Grove Street, Cambridge, MA 02138 (US). SCHMITZ, Tracy; 475 Huron Avenue, Cambridge, MA 02138 (US). UNDERWOOD, Robert; 93 Walker Street, Quincy, MA 02171 (US). <b>(74) Agent:</b> PLUMER, Elizabeth, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> USE OF CD26 INHIBITOR FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF HIV  <b>(57) Abstract</b>  The use of a molecule that inhibits CD26 for the preparation of a medicament for increasing the immune response of a human patient infected with HIV, by contacting the T-cells, <i>in vitro</i> or <i>in vivo</i> , with the said molecule at a concentration effective to cause T-cell proliferation, but below an amount that causes detectable cytotoxicity.		

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# USE OF CD26 INHIBITOR FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF HIV

## Government Support

5           This work was funded in part by grant number A136696 from the National Institutes of Health. Accordingly, the United States Government may have certain rights to this invention.

## Cross-Reference to Related Applications

          This application claims priority from U.S. Patent Application Serial No. 08/852,394,  
10   filed on May 7, 1997, entitled TREATMENT OF HIV. The contents of this U.S. Patent application are hereby expressly incorporated by reference.

## Background of the Invention

          This invention relates to treatment of HIV.

15           One of the classic markers of full-blown AIDS resulting from long-term infection with HIV-1 is a severe depletion of CD4<sup>+</sup> T-cells, which are a key component of the immune system. Attempts have been made to increase the CD4<sup>+</sup> counts of AIDS patients, and some of these efforts, notably treatment with HIV protease inhibitors, have met with considerable success. Other approaches, e.g., stimulation of the immune response by vaccination with viral peptides,  
20   have been less successful. The reasons for CD4<sup>+</sup> depletion in AIDS, and resistance of CD4<sup>+</sup> cells to stimulation by some therapies, are not fully understood.

## Summary of the Invention

          The invention provides a new and highly advantageous method of potentiating the  
25   immune response in HIV-infected patients, employing extremely low concentrations of compounds which, at these concentrations, act as stimulatory, rather than inhibitory, molecules. The very low concentrations employed according to the invention allow treatment with minimal side effects and toxicity. The specificity of the treatments according to the invention also helps avoid these adverse effects, which are seen for example, in treatment with immune  
30   stimulatory compounds such as Interleukin-2.

          The invention involves the unexpected finding that certain compounds, which at relatively low concentrations (e.g., 10<sup>-4</sup>-10<sup>-6</sup>M) are cytotoxic to T-cells, nevertheless have immune stimulatory properties at extremely low concentrations (e.g., 10<sup>-10</sup>-10<sup>-14</sup>M). Even more

surprising, this stimulation occurs even though the HIV-infected patient's T-cells are otherwise unable to respond to T-cell proliferation-inducing stimuli. The effects of these low concentrations are paradoxical because T-cells from non-HIV-infected individuals, which  
5 respond to T-cell proliferation-inducing stimuli, do not seem to respond in the same fashion as T-cells from a HIV-infected patient when treated with the extremely low concentrations of compounds according to the invention.

The invention thus provides a method for treating the T-cells of a human subject infected with human immunodeficiency virus. The subject's T-cells are contacted with a  
10 molecule that inhibits CD26 and that stimulates immune function of the T-cells in an amount effective to stimulate immune function of the T-cells, said amount being below a concentration which causes detectable cytotoxicity of the T-cells. In one embodiment, the molecule stimulates proliferation of T-cells at the effective concentration. In other embodiments, the molecule stimulates the production of cytokines or increases cytotoxic T-lymphocyte or  
15 antibody activity.

The T-cells can be contacted *in vitro* or *in vivo*. In certain embodiments, the effective amount is below  $10^{-8}$ M and may be between  $10^{-10}$  and  $10^{-16}$ M. Molarity is measured as a function of final concentration *in vitro* and as a function of blood concentration *in vivo*.

According to another aspect of the invention, the molecule can be administered in  
20 conjunction with a different therapeutic agent that increases the CD4<sup>+</sup> count of HIV-infected subjects. In this manner, treatment with effective amounts of the molecule according to the invention can enhance the therapeutic effect of other AIDS drugs. Specifically contemplated is use of the molecules according to the invention with non-CD26 protease inhibitors. It has been determined that the effects of the treatments according to the invention are particularly good in  
25 patients whose CD4<sup>+</sup> count is above about 400. In one embodiment, subjects may be treated with therapeutic agents or regimens to increase T-cell count to above 400 where the count initially is below 400. The subjects then are believed to be better candidates for treatment according to the invention. Thus, the invention contemplates the use of the molecules according to the invention to provide optimal combination AIDS therapies. Particularly  
30 contemplated for use in conjunction with the molecules of the invention are therapeutics which inhibit HIV replication by, for example, inhibiting reverse transcriptase or by inhibiting HIV protease activity. Exemplary therapeutics include the antiretroviral drugs: AZT (3' azido-2',3'

dideoxythymidine), ddI (2',3' dideoxyinosine), ddC(2',3' dideoxycytidine), ddT (2',3' dihydro-3'-deoxythymidine) and other nucleoside and non-nucleoside reverse transcriptase inhibitors and HIV-1 protease inhibitors: Indinavir, Ritonavir, Saquinavir. Combination therapies include the administration of one or more of these or other antiretroviral therapeutics, alone or together with therapeutics that are intended to treat the secondary infections associated with HIV and/or cytokines (e.g., GM-CSF, G-CSF, interferons, interleukins).

According to another aspect of the invention, the molecules of the invention are contacted with T-cells of a human subject infected with HIV, which T-cells are unable, prior to treatment according to the invention to respond normally to T-cell proliferation-inducing stimuli. In one particularly important embodiment, the molecules of the invention are administered in conjunction with an antigen. Ordinarily, such HIV-infected patients do not respond well to antigens because of an HIV-induced defect in the T-cell stimulation pathway. Use of the molecules according to the invention as adjuvants can render such T-cells responsive to stimulation or vaccination with antigens. The invention permits immunization of HIV-infected patients with antigens characteristic of HIV, antigens characteristic of other pathogens, antigens characteristic of cancer cells and the like. Peptide antigens in particular are contemplated.

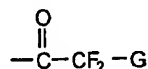
Compounds useful in the invention inhibit CD26 and stimulate proliferation of T-cells of HIV-infected subjects in the assays and at the concentrations described below. CD26 is also referred to as "dipeptidyl-aminopeptidase type-IV" or "DP-IV". CD26 is a post-prolyl cleaving enzyme with a specificity for removing Xaa-Pro (where Xaa represents any amino acid) dipeptides from the amino terminus of a polypeptide substrate.

Peptides which reportedly have demonstrated utility for inhibiting post-prolyl cleaving enzymes and which, if coupled to a reactive group, form a covalent complex with a functional group in the reactive site of a post-prolyl cleaving enzyme are described in U.S. Patent No. 4,935,493, "Protease Inhibitors", issued to Bachovchin et al. ("Bachovchin '493"); U.S. 5,462,928, "Inhibitors of Depeptidyl-aminopeptidase Type IV", issued to Bachovchin et al. ("Bachovchin '928"); U.S. 5,543,396, "Proline Phosphonate Derivatives", issued to Powers et al. ("Powers '396"); U.S. 5,296,604, "Proline Derivatives and Compositions for Their Use as Inhibitors of HIV Protease", issued to Hanko et al., ("Hanko '604"); PCT/US92/09845, "Method for Making a Prolineboronate Ester", and its U.S. priority applications (USSN 07/796,148 and 07/936,198), Applicant Boehringer Ingelheim Pharmaceuticals, Inc.

("Boehringer"). Representative structures of the transition-state analog-based inhibitors Xaa-Boro-Pro, include Lys-boroPro, Pro-boroPro, Val-boroPro and Ala-boroPro in which "boroPro" refers to the analog of proline in which the carboxylate group (COOH) is replaced with a boronyl group (B) (OH<sub>2</sub>).

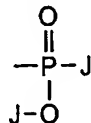
5 In general, the molecules useful according to the invention can have a first targeting moiety for binding a post-prolyl cleaving enzyme such as CD26 covalently coupled to a first reactive group. As used herein, a reactive group is capable of reacting with a functional group in a post-prolyl cleaving enzyme such as CD26. By reacting, it is meant that the reactive group forms a bond with a functional group of a post-prolyl cleaving enzyme such as CD26. Reactive  
10 groups that are embraced within the invention include the reactive groups referred to as group "T" in U.S. 4,935,493, "Protease Inhibitors", issued to Bachovchin, et al. These include boronate groups, phosphonate groups, and fluoroalkylketone groups. In general, it is preferred that the linkage between the carboxyl terminus of preferred peptide targeting moieties and the reactive group be in an L configuration. It is preferred that the reactive group form a covalent  
15 bond with a functional group of the active site of a reactive center of CD26; however, there is no requirement for covalent bond formation in order to form a complex between the CD26 binding molecule and CD26.

The reactive groups that are fluoroalkylketone groups have the formula:



20 where G is either H, F or an alkyl group containing 1 to about 20 carbon atoms and optional heteroatoms which can be N, S, or O. Additional exemplary proline phosphonate derivatives which contain a perfluoroalkyl group, a phenyl group or a substituted phenyl group and which can be used in accordance with the methods of the invention are those described in U.S. 5,543,396 (Powers '396).

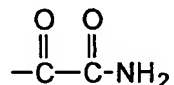
25 The reactive groups that are phosphonate groups have the formula:



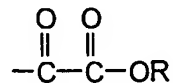
where each J, independently, is O-alkyl, N-alkyl, or alkyl (each containing about 1-20 carbon atoms) and, optionally, heteroatoms which can be n=N, S, or O. Other ketoamides, ketoacids

and ketoesters that are useful reactive groups for reacting with the reactive center of a protease are described in PCT/US91/09801, "Peptides, Ketoamides, Ketoacids, and Ketoesters", Applicant: Georgia Tech Research Corp. ("GA Tech") which claims priority to U.S. 635,287, filed December 28, 1990.

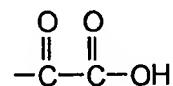
- 5 In certain embodiments, the reactive groups are selected from the groups having the formulas:



an alphaketo amide;



- 10 where R is an alkyl, or aryl group and may be substituted or unsubstituted, an alphaketo ester; and



an alphaketo acid.

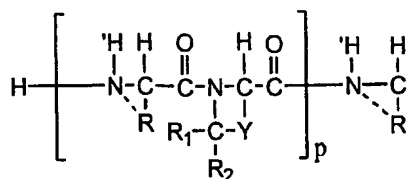
- The preferred compounds having targeting moieties that are peptides which mimic the substrate binding site of DP-IV. Peptide analogs and nonpeptides or peptidomimetics also can be used as targeting moieties. Such molecules can be rationally designed based upon the known sequence of substrates of DP-IV or can be identified using combinatorial chemistry and screening assays such as are described below.

- The development of phage display libraries and chemical combinatorial libraries permits the selection of synthetic compounds which mimic the substrate binding site of a protease such as CD26. Such libraries can be screened to identify non-naturally occurring putative targeting moieties by assaying protease cleavage activity in the presence and absence of the putative phage display library molecule or combinatorial library molecule and determining whether the molecule inhibits cleavage by the protease of its natural substrate or of a substrate analog (e.g., a chromophoric substrate analog which is easily detectable in a spectrophotometric assay). Those phage library and/or combinatorial library molecules which exhibit inhibition of the protease then can be covalently coupled to the reactive groups R disclosed herein and again tested to determine whether these novel molecules selectively bind

to the protease (e.g., by repeating the above-noted screening assay). In this manner, a simple, high-through-put screening assay is provided for identifying non-naturally occurring targeting moieties of the invention.

Compounds useful in the invention include, but are not limited to, compounds that inhibit CD26 and are embraced by the following formula PR, wherein P represents a targeting moiety that binds to CD26 and R represents a reactive group that reacts with a functional group in CD26, preferably a reactive center of CD26. P can be any molecule that binds CD26 including CD26 binding molecules embraced by the formula: D~A<sub>1</sub>-A<sub>2</sub>-A<sub>3</sub>-A<sub>4</sub>, wherein D is independently selected from the group consisting of NH and NH<sub>2</sub>, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~", independently, is selected from the group consisting of a single bond and a double bond; A<sub>1</sub> is selected from the group consisting of a C, a CX and an N, wherein C represents any isotope of carbon, X represents any atom that forms a single bond with carbon; each A<sub>2</sub>, A<sub>3</sub>, and A<sub>4</sub>, independently, is selected from the group consisting of a CX moiety, a CXZ moiety, a CZ moiety, a NX moiety, and an O, wherein X and Z, independently are selected from the group consisting of any atom that forms a single bond and any atom that forms a double bond with C or N and wherein O represent any isotope of oxygen.

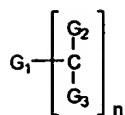
Compounds useful according to the invention also include the following of Group I or Group II. Group I has the structure:



where H represents a hydrogen; C represents a carbon; O represents an oxygen; N represents a nitrogen; each R, independently, is chosen from the group consisting of the R groups of an amino acid, including proline; each broken line, independently, represents a bond to an H or a bond to one R group, and each 'H represents that bond or a hydrogen; and p is an integer between 0 and 4 inclusive. Alternatively, Group I has the structure:

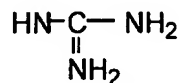


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where n is between 0 and 3 inclusive, each G2 and G3 independently is H or Cl-C3 (one to three carbon atoms) alkyl, G1 is NH<sub>3</sub> (H<sub>3</sub> represents three hydrogens),

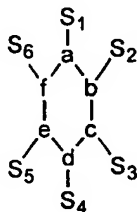
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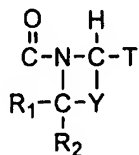
(H<sub>2</sub> represents two hydrogens), or NG4, where G4 is



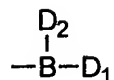
where G5 and G6 can be NH, H, or Cl-C3 alkyl or alkenyl with one or more carbons substituted  
10 with a nitrogen. G1 bears a charge, and G1 and Group II do not form a covalently bonded ring structure at pH 7.0. Group I may also have the structure:



where one or two of the a, b, c, d, e, and f group is N, and the rest are C, and each S1-S6  
15 independently is H or Cl-C3 alkyl. Group I may also include a five membered unsaturated ring having two nitrogen atoms, e.g., an imidazole ring. Group II has the structure:

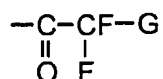


where T is a group of the formula:



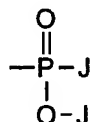
where each D<sub>1</sub> and D<sub>2</sub>, independently, is a hydroxyl group or a group which is capable of being hydrolyzed to a hydroxyl group in aqueous solution at physiological pH; a group of the

5 formula:



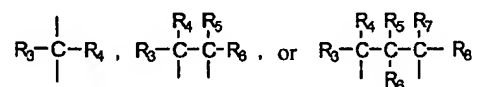
where G is either H, fluorine (F) or an alkyl group containing 1 to 20 carbon atoms and optional heteroatoms which can be N, S (sulfur) or O; or a phosphonate group of the formula:

10



where each J, independently, is O-alkyl, N-alkyl, or alkyl. Each O-alkyl, N-alkyl or alkyl includes 1-20 carbon atoms and, optionally, heteroatoms which can be N, S, or O. T is generally able to form a complex with the catalytic side of a DP-IV. Y is:

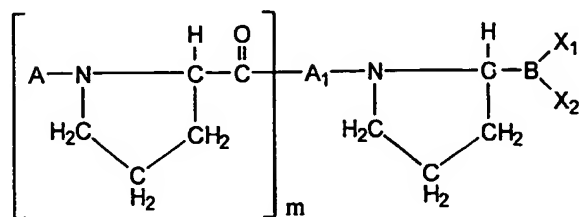
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and each R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub>, R<sub>6</sub>, R<sub>7</sub>, and R<sub>8</sub>, separately is a group which does not significantly interfere with site specific recognition of the inhibitory compound by DP-IV, and allows a complex to be formed with DP-IV.

In preferred embodiments, T is a boronate group, a phosphonate group or a trifluoroalkyl ketone group; each R<sub>1</sub>-R<sub>8</sub>, is H; each R<sub>1</sub> and R<sub>2</sub> is H, and each Y is the CH<sub>2</sub>-CH<sub>2</sub>; each R is independently chosen from the R group of proline and alanine; the inhibitory compound has a binding or dissociation constant to DP-IV of at least 10<sup>-9</sup>M, 10<sup>-8</sup>M or even 10<sup>-7</sup>M; and each D<sub>1</sub> and D<sub>2</sub> is, independently, F or D<sub>1</sub> and D<sub>2</sub> together are a ring containing 1 to 20 carbon atoms, and optionally heteroatoms which can be N, S, or O. These compounds are  
25 described in U.S. Patent No. 5,462,928, hereby incorporated by reference.

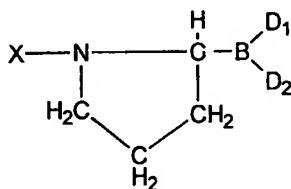
More preferably, the compound used at low concentration according to the invention has the formula:



wherein m is an integer between 0 and 10, inclusive; A and A<sub>1</sub> are amino acid residues such  
 5 that the A in each repeating bracketed unit can be a different amino acid residue; the bonds  
 between A and N, A<sub>1</sub> and C, and between A<sub>1</sub> and N are peptide bonds; and each X<sub>1</sub> and X<sub>2</sub> is,  
 independently, a hydroxyl group or a group capable of being hydrolyzed to a hydroxyl group at  
 physiological pH. These compounds are also described in U.S. Patent No. 5,462,928.

The most preferred compounds are of the formula:

10



where each D<sub>1</sub> and D<sub>2</sub>, independently, is a hydroxyl group or a group which is capable of being  
 hydrolyzed to a hydroxyl group in aqueous solution at physiological pH;

and wherein X is a targeting moiety that mimics the site of a substrate recognized and  
 15 cleaved by CD26.

Other features and advantages of the invention will be apparent from the following  
 detailed description thereof, and from the claims.

### **Brief Description of the Drawings**

20 Fig. 1 is a pair of graphs showing the lymphocyte stimulatory effect of treatment of the  
 invention on peripheral blood mononuclear cells (PBMC) from HIV-infected and uninfected  
 patients. Fig. 1A shows the effect of the compound on T-cell proliferation *in vitro* for PBMC from  
 an HIV-1<sup>+</sup> individual and Fig. 1B shows the effect of the compound on T-cell proliferation *in vitro*

on PBMC from an HIV-1<sup>+</sup> individual. Each of Figs. 1A and 1B illustrate a representative experiment out of a total of ten experiments.

Fig. 2 is a graph illustrating the T-cell stimulatory effects of two inhibitory compounds used according to the invention (date of experiment: 3/9/95; Patient ID no:1655185; CD4 antibody count:760; and number of cells/well:  $0.4 \times 10^6$ ).

Fig. 3 is a histogram demonstrating that an inhibitor according to the invention induces, at higher doses than in the invention, dose-dependent apoptosis in both CD26<sup>+</sup> and CD26<sup>-</sup> populations of PBMC. The CD26<sup>+</sup> and CD26<sup>-</sup> PBMC populations were found to be equally susceptible to DPPIV inhibitor induced death. PBMC were stained with the anti-CD26 monoclonal antibody, 4 EL, and then sorted into CD26<sup>+</sup> and CD26<sup>-</sup> populations using a facstar plus dual laser flow cytometry. The cells expressing the highest level (5%) of CD26 and the cells expressing the lowest level (bottom 10%) of CD26 were isolated as the CD26<sup>+</sup> and CD26<sup>-</sup> populations respectively. The purity of the populations as examined by staining with the anti-CD26 monoclonal antibody, 134-2C2, is >90%. The CD26<sup>+</sup> and CD26<sup>-</sup> populations were cultured overnight in the presence or absence of various concentrations of VBP. The amount of death induced by VBP treatment was determined by 7AAD flow cytometry analysis. Data represent mean of death from duplicate samples +/-SD.

Fig. 4 is a graph illustrating a stimulatory effect of an inhibitor according to the invention on PBMC *in vitro*, showing the correlation with CD4<sup>+</sup> counts. The data are plotted as the natural log of the stimulation index (vertical dimension) versus the natural log of the CD4<sup>+</sup> count of the patient (horizontal dimension)(71 patients total;  $P < 0.0001$ ;  $RR = 2.04$  (1.5-2.9)).

Figs. 5 and 6 are graphs showing T-cell stimulatory effect on lymphocytes from HIV-infected patients by a CD26 inhibitor according to the invention, and giving results for control compounds.

#### **Detailed Description of the Invention**

In general, the compounds useful according to the invention are capable of stimulating immune function of T-cells from HIV-infected human subjects. Stimulation of immune function is preferably determined by measuring whether T-cells proliferate in response to treatment according to the invention. Assays for proliferation are described below. Stimulation of immune function also can be determined by detecting an increase in cytokine release or by detecting an increase in cytotoxic T-lymphocyte or antibody activity, using routine, commercially available

assays and/or reagents.

The molecules are contacted with T-cells in amounts effective to stimulate immune function. Effective amounts are those which cause detectable changes in any one or more of the foregoing parameters. Thus, for example, an effective amount can be that amount which induces measurable proliferation of an HIV-infected subject's T-cells. The effective amounts according to the invention, however, are amounts below a concentration which causes detectable cytotoxicity of a patient's T-cells. As used herein, detectable cytotoxicity means no more than 20%, preferably no more than 10% and most preferably no more than 5% cytotoxicity as compared to controls. At such levels, the proliferation effects of the compounds will outweigh the cytotoxic effects.

An important aspect of the invention is the discovery that relatively high concentrations (e.g.,  $10^{-4}$ - $10^{-6}$  M) of certain compounds useful according to the invention cause substantial cytotoxicity of T-cells. For example, using Val-boroPro, greater than 80% toxicity was observed *in vitro* at a concentration of  $10^{-4}$ M and approximately 50% cytotoxicity was observed at a concentration of  $10^{-6}$ M. Although cytotoxicity decreased with decreasing concentrations of Val-boroPro, it was not expected that there would be such substantial cytotoxicity at concentrations of  $10^{-6}$ . Unexpectedly, however, stimulatory properties increased substantially at concentrations significantly below those concentrations which were cytotoxic. Maximum stimulation was observed at concentrations of  $10^{-12}$ - $10^{-14}$ M. Thus, the compounds according to the invention can be applied at extremely low concentrations *in vitro*, or *in vivo*, thereby avoiding any detectable cytotoxicity yet achieving the highest levels of immune stimulation.

The foregoing observation is important when considering treatments of T-cells of HIV-infected subjects, particularly *in vivo*. AIDS subjects already suffer drastic reductions in T-cells. It could be catastrophic to administer compounds which would destroy the relatively meager amount of T-cells present in an AIDS patient. The present invention is based upon the discovery that concentrations of certain compounds can be applied far below those concentrations that would be cytotoxic while achieving the highest levels of immune stimulation capable of being induced by such compounds.

In selecting effective amounts for *in vivo* treatment, concentrations can be selected far outside the range that would be expected to cause cytotoxicity. This concentration then could be increased, all the while measuring T-cell count, until maximum stimulation is observed (as in a bell curve). Maximum stimulation will occur, as shown in the Examples below, long before cytotoxic levels are achieved. Thus, effective *in vivo* dosing can be accomplished without risk to the patient.

In selecting amounts for *in vitro* treatment, assays such as those shown in the Examples below can be used. One such assay employs the candidate compound at very low concentrations, in a test designed to determine whether at such concentrations the compound can stimulate proliferation of PBMC from HIV-infected patients *in vitro*. As is shown in the Examples, stimulation can be measured by, e.g., incorporation of a labeled nucleotide. *In vitro* screening also can be used to assist in developing *in vivo* effective amounts.

Where *in vitro* treatment is contemplated, cells are obtained from a subject, stimulated *in vitro*, and then returned to the subject in a conventional manner. Where *in vivo* treatment is contemplated, the compounds useful according to the invention are administered in a conventional manner, as described in greater detail below.

The molecules useful according to the invention can be administered *in vivo* as concurrent therapy or in combination with other AIDS therapeutics. While not wishing to be bound by any theory of the invention, it is believed that the compounds of the invention will beneficially stimulate proliferation of *non-infected* T-cells in the HIV-infected patient. The concurrent treatments then can be used to bring about proliferation of non-infected T-cells, which in turn can be stimulated beneficially according to the methods of the present invention. The fact that, in T-cells treated according to the invention *in vitro* no increase in the level of the HIV protein p24 is observed, may indicate that the T-cells which are infected with HIV are not stimulated by the low dose treatment of the invention. The inventors explicitly, however, do not intend to be bound by this theory of the invention.

Particularly preferred compounds useful according to the invention are described in the Examples below. The invention, however, embraces the specific compounds described above. In order to identify additional compounds useful according to the invention, simple screening assays can be employed. First, compounds rationally designed based upon the natural substrate of a post-prolyl cleaving enzyme or libraries of compounds prepared by combinatorial chemistry or phage display are screened for their ability to inhibit CD26. CD26 and methods of purifying CD26 are well-known to those of ordinary skill in the art. Torimoto, Y., et al., *Molecular Immunol.* 29:183-192 (1992). Assays which measure, for example, the ability of a molecule to interfere with CD26 cleavage of a standard fluorescent-labeled substrate can be employed. Once a candidate compound that inhibits CD26 is identified, then the compound is tested over a wide range of concentrations (e.g.,  $10^{-4}$ - $10^{-16}$  M) to determine the ability of the compound to stimulate immune function of T-cells from HIV-infected patients. Methods for performing this test are described in the Examples

below, which involve measurement of proliferation.

In the screening assays described in the previous paragraph, a reporter substrate such as Ala-Pro-AFC, which contains a proline in the penultimate position, can be employed. Alternatively, a colorimetric assay can be carried out using as a substrate Gly-Pro-PNA. The  
5 choice of terminal amino acid is not critical, provided that the substrate contain a free terminal amino group. A fluorescence spectrometer then can be used to measure cleavage of the reporter substrate. Multiple samples can be run, with or without a test compound. The samples are placed in a cuvette, inserted into a fluorescent spectrometer, and enzymatic activity is measured as the accumulation of fluorescence intensity (i.e., substrate cleavage product) over time (e.g., 1 minute).  
10 A compound is identified as a good candidate if the accumulative fluorescence is decreased as a result of the presence of the compound.

When methods are carried out *in vivo*, the effective amount will vary with the particular condition being treated, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent therapy (if any), the  
15 specific route of administration and the like factors within the knowledge and expertise of the health practitioner. For example, an effective amount can depend upon the degree to which an individual has abnormally depressed levels of T-cells.

Generally, doses of active compounds would be from about 0.001 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from .01-10 mg/kg will be suitable, preferably  
20 orally and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits, provided that toxic levels are not administered. Multiple doses per day are contemplated to achieve  
25 appropriate systemic levels of compounds.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. Such preparations may routinely contain salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically-  
30 acceptable, but non-pharmaceutically-acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically- and pharmaceutically-acceptable salts include, but are not limited to,

those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

5           The molecules may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the  
10       pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

          The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

15           The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

          Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the anti-inflammatory agent, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable  
20       dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For  
25       this purpose any bland fixed oil may be employed using synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

          A variety of administration routes are available. The particular mode selected will depend,  
30       of course, upon the particular drug selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that



produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Oral administration will be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active agent. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the active agent, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di- and tri-glycerides; hydrogel release systems; sytastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the active agent is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,667,014, 4,748,034 and 5,239,660 and (b) diffusional systems in which the active agent permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,832,253 and 3,854,480. In addition, pump-based hardware

delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably  
5 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

Certain families of molecules useful according to the invention are described in U.S. Patent Nos. 4,935,493, 5,462,928, and in U.S. Application Serial No. 07/923,337, corresponding to PCT Application No. WO94/03055. One of the families of molecules described in these patents may  
10 be referred to as the Xaa-Boro-Pro molecules, exemplified by Ala-boroPro, Pro-boroPro, and Gly-boroPro. Two of these compounds, used in the Examples described below, are Lys-Boro-Pro ("KBP") and Val-Boro-Pro ("VBP").

### EXAMPLES

15 Peripheral blood mononuclear cells (PBMC) were obtained by standard methods from HIV-infected individuals, and from uninfected individuals. Varying dosages of KBP or VBP were contacted with the PBMC *in vitro*, and stimulation of proliferation was measured by incorporation of  $^3\text{H}$  thymidine (cpm). The results of these experiments are shown in Fig. 1: very low doses of the Val-Boro-Pro and Lys-Boro-Pro stimulated proliferation of PBMC from HIV-infected patients,  
20 but not PBMC from uninfected patients.

As shown in Fig. 1, at no concentration tested of the compounds did they affect the PBMC from uninfected individuals. The compounds, at moderate concentrations, also did not cause proliferation of PBMC from HIV-infected individuals, but did cause marked proliferation at very low concentrations ( $10^{-9}$  and  $10^{-10}\text{M}$ ). The same results are shown in Figs. 5 and 6 which also  
25 present data for two control compounds OKT3 and PHA, both of which are non-specific mitogens.

Fig. 2 is a histogram showing that relatively low doses of Lys-boroPro and Val-boroPro ( $10^{-7}\text{M}$ ) caused proliferation of PBMC of HIV-infected patients, while higher doses ( $10^{-4}\text{M}$ ) did not have this effect. This is to be contrasted with Figure 3 which shows that Val-boroPro is highly cytotoxic to T-cells at concentrations of  $10^{-4}$  and  $10^{-6}\text{M}$ . Thus, even though there was stimulation  
30 at concentrations of  $10^{-6}$  as shown in Fig. 2, it would be undesirable to use Val-boroPro in this range because it would cause T-cell death in patients already severely compromised with T-cell loss. As shown in Figs. 5 and 6, Val-boroPro can be used successfully outside of this cytotoxic

range, avoiding the cytotoxic effects.

Referring to Fig. 4, data are presented in a form showing that low concentrations of the compounds of the invention have less effect on the PBMC of HIV-infected patients whose CD4<sup>+</sup> counts are lower than about 400 (clinical indication for AIDS) than on the PBMC of HIV-infected  
5 patients whose CD4<sup>+</sup> counts are higher than about 400. In the graph of Fig. 5, the natural log of the stimulation index (the vertical axis) is plotted against the natural log of the CD4<sup>+</sup> count of the patients; as is shown.

The disclosures of any patents and patent applications referenced to herein are expressly incorporated by reference.

10 We claim:

**CLAIMS**

1. A method for treating the T-cells of a human subject infected with Human Immunodeficiency Virus, comprising:

contacting said T-cells with a molecule that inhibits CD26 and that stimulates immune  
5 function of said T-cells in an amount effective to stimulate immune function of the T-cells, said amount being below a concentration which causes detectable cytotoxicity of said T-cells.

2. The method of claim 1, wherein said molecule stimulates proliferation of said T-cells at said effective concentration.

10

3. The method of claim 1, wherein the T-cells are contacted *in vitro* and then are administered to the subject.

4. The method of claim 1, wherein the T-cells are contacted *in vivo*.

15

5. The method of claim 3, wherein the effective amount is below  $10^{-8}$ M.

6. The method of claim 4, wherein the effective amount is a blood concentration below  
10<sup>-8</sup>M.

20

7. The method of claim 5, wherein the effective amount is between  $10^{-10}$  and  $10^{-16}$ M.

8. The method of claim 6, wherein the effective amount is a blood concentration between  $10^{-10}$  and  $10^{-16}$ M.

25

9. The method of claim 4, wherein the molecule is administered in conjunction with a different therapeutic agent that increases the CD4<sup>+</sup> count of HIV-infected patients.

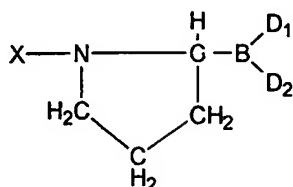
10. The method of claim 1, wherein the human subject's T-cells are unable, prior to  
30 treatment with said molecule, to respond normally to T-cell proliferation-inducing stimuli.

11. The method of claim 4, wherein the molecule is administered in conjunction with

an antigen.

12. The method of claims 1-11, wherein the molecule mimics the site of a substrate recognized by a post-prolyl cleaving enzyme and includes a reactive group that binds covalently  
5 with a functional group in a reactive center of the post-prolyl cleaving portion of CD26.

13. The method of claim 12, wherein the molecule has the formula:



- 10 wherein X is a targeting moiety that mimics the site of a substrate recognized and cleaved by CD26.

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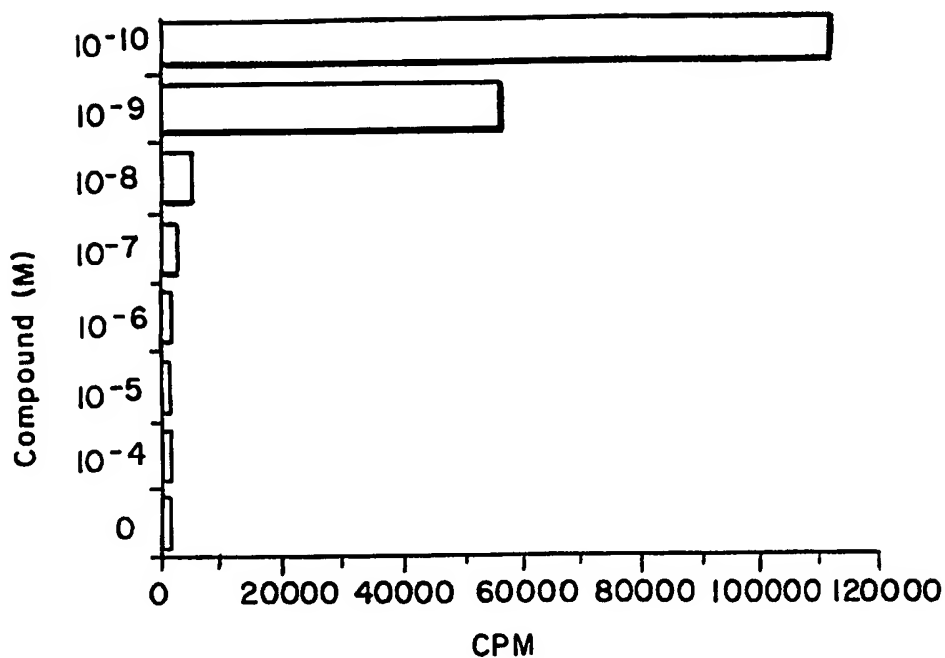


FIG. 1A

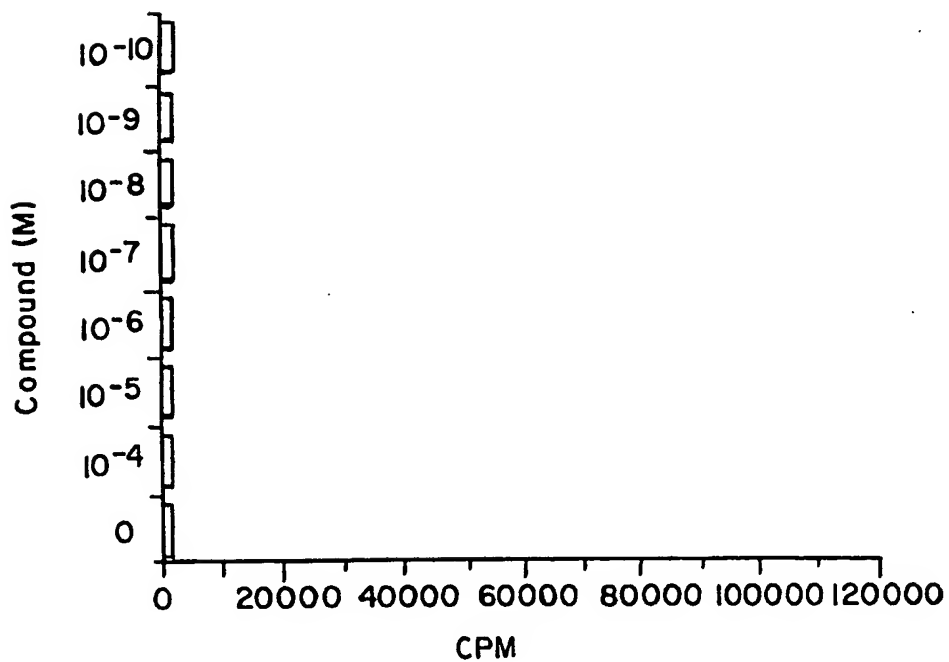


FIG. 1B

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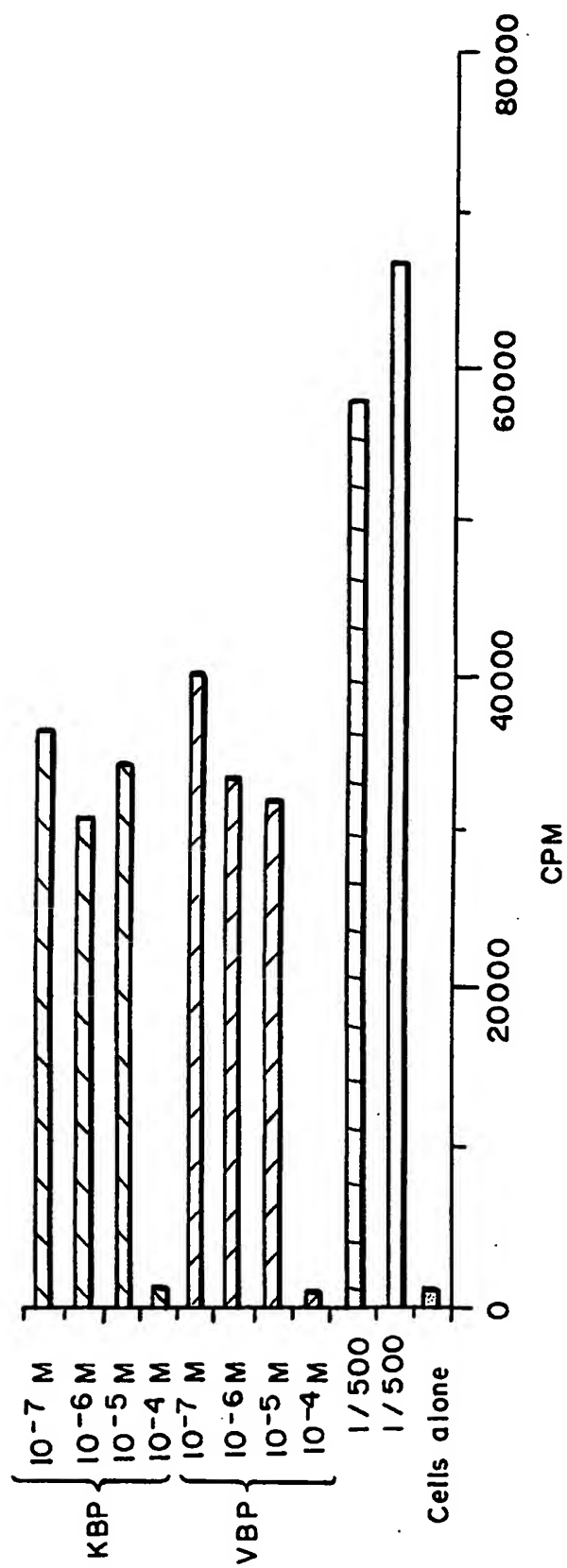


FIG. 2

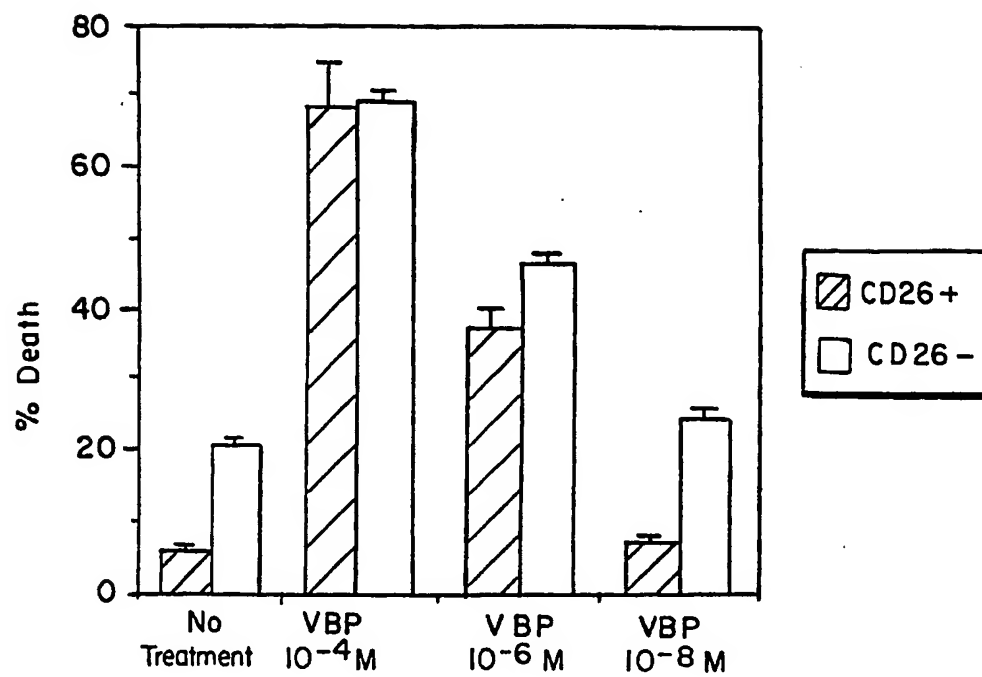


FIG. 3



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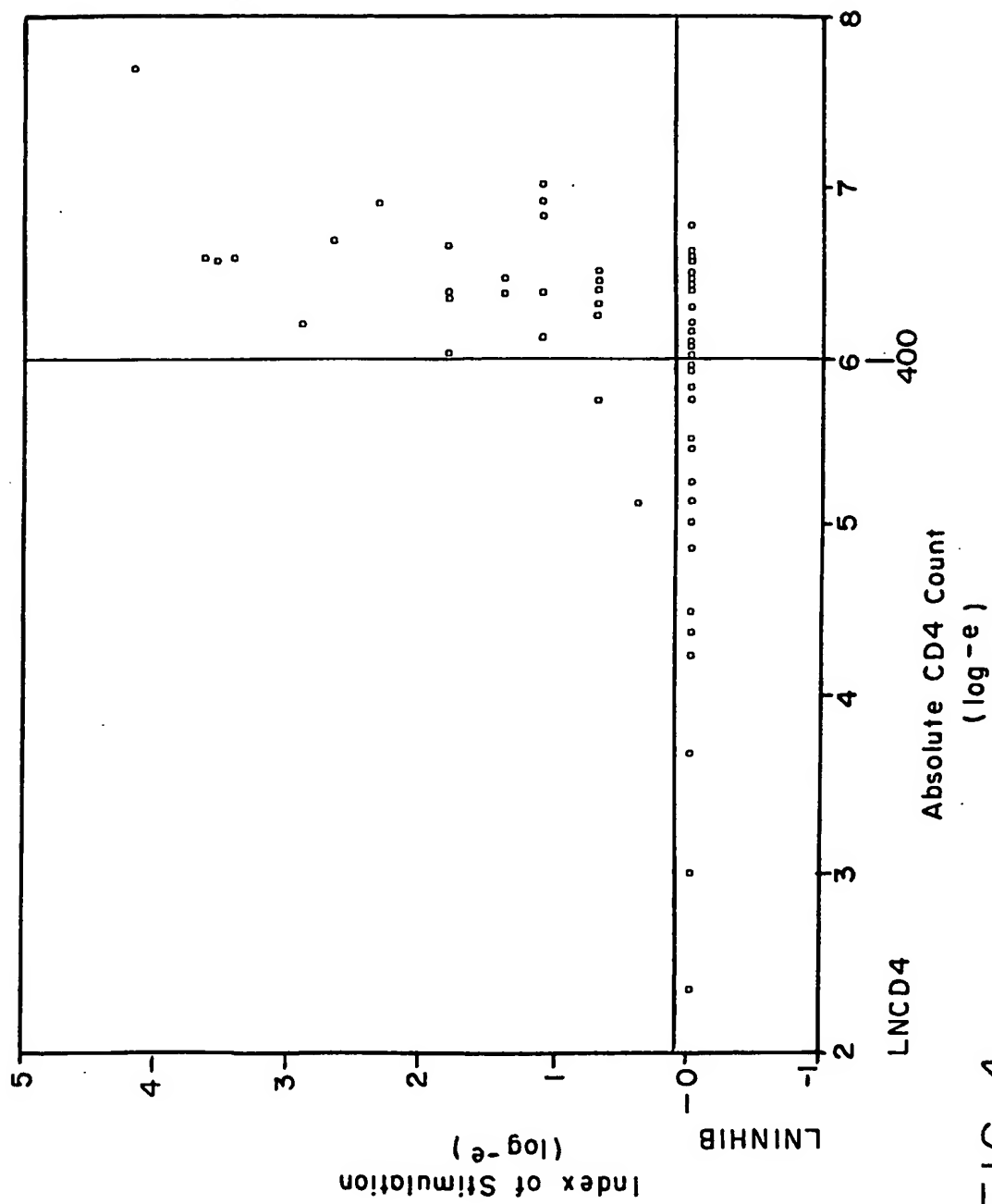


FIG. 4

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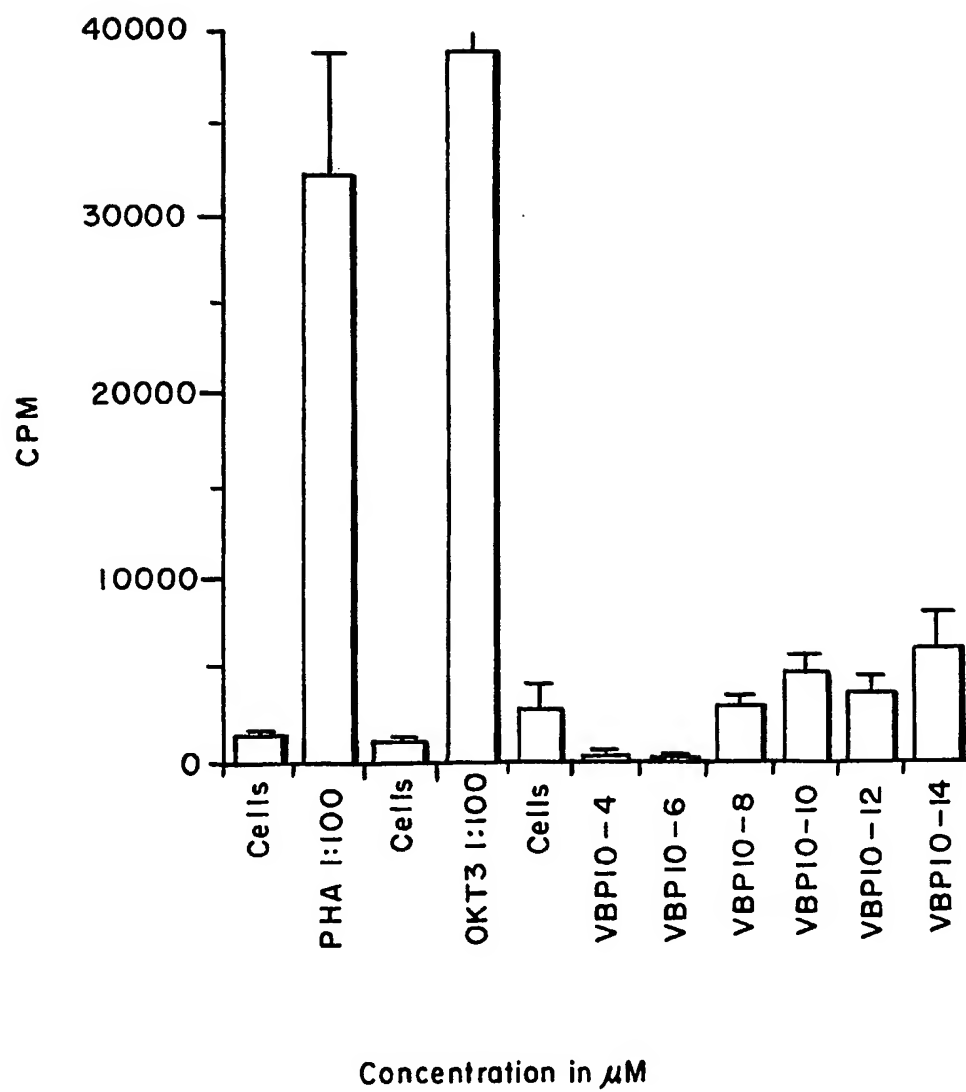


FIG. 5

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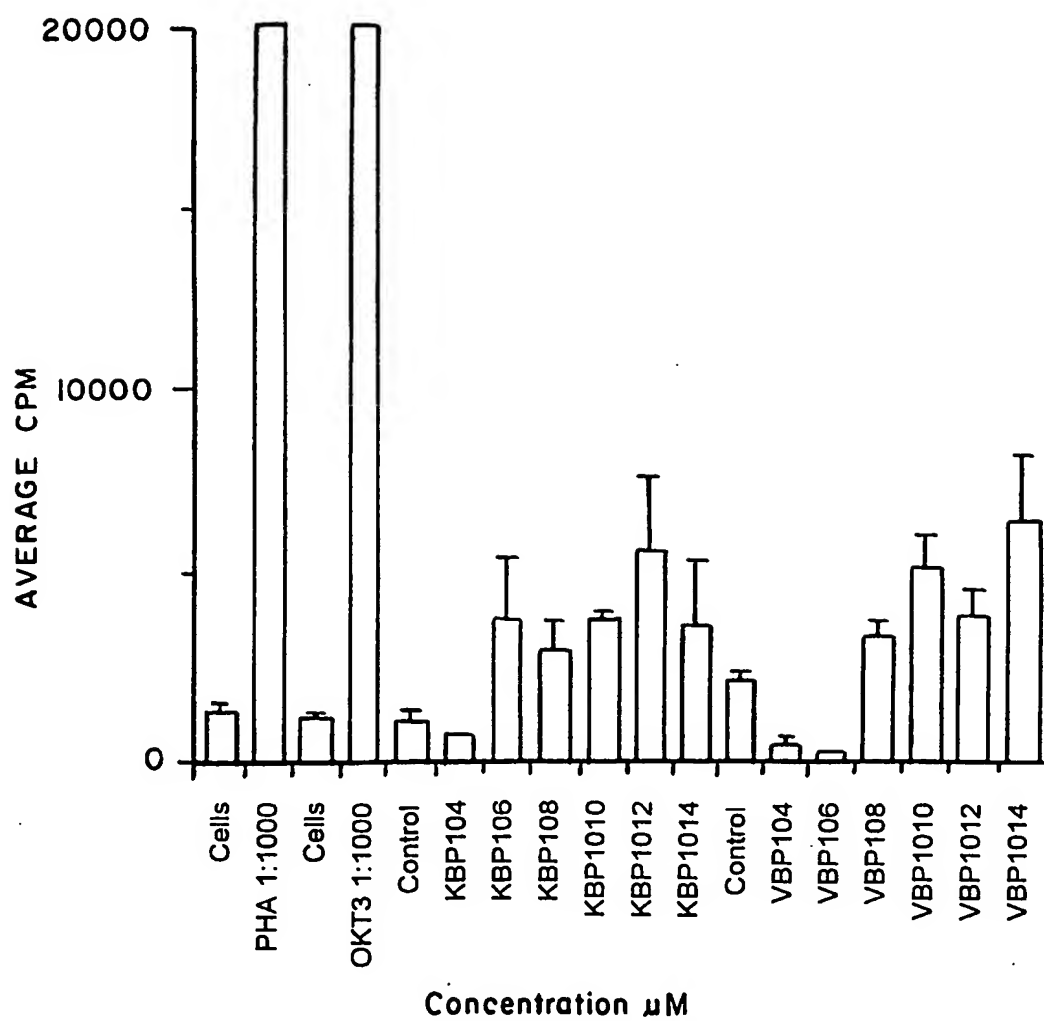


FIG. 6

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/08723

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K31/69 A61K38/55

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 03055 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA)) 17 February 1994 cited in the application see page 1-12 see claims 1-26 ---	1-13
X	WO 95 29691 A (GEORGIA TECH RESEARCH CO.) 9 November 1995 cited in the application see the whole document ---	1-13
X	WO 89 03223 A (BACHOVCHIN W.W.) 20 April 1989 cited in the application see the whole document ---	1-13
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

10 August 1998

Date of mailing of the international search report

01/09/1998

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# INTERNATIONAL SEARCH REPORT

Int lional Application No

PCT/US 98/08723

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 08259 A (NEW ENGLAND MEDICAL CENTER HOSPITALS INC.) 29 April 1993 cited in the application see the whole document ---	1-13
X	US 5 296 604 A (MILES INC.) 22 March 1994 cited in the application see the whole document ---	1-13
X	WO 92 12140 A (GEORGIA TECH RESEARCH CO.) 23 July 1992 cited in the application see the whole document ---	1-13
X	COUTTS S.J. ET AL.: "Structure-Activity Relationships of Boronic Acid Inhibitors of Dipeptidyl Peptidase IV.1. Variation of the P2 Position of Xaa-boroPro Dipeptides" J.MED.CHEM., vol. 39, no. 10, 10 May 1996, USA, pages 2087-2094, XP002074031 see page 2087, left-hand column, line 1 - page 2087, right-hand column, paragraph 2 see abstract ---	1-13
P,X	JIANG J.D. ET AL.: "Inhibition of uman immunodeficiency virus type 1 infection in a T-cell line (CEM) by new dipeptidyl-peptidase IV (CD26) inhibitors" RES.VIROL, vol. 148, no. 4, July 1997 - August 1997, FRANCE, pages 255-266, XP002074032 see page 258, left-hand column, paragraph 5 - page 264, left-hand column, line 41 see figure 1 see page 255, left-hand column, line 1 - page 256, right-hand column, line 20 see abstract -----	1-13

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/08723

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9403055	A	17-02-1994	AU 4794393 A	03-03-1994
WO 9529691	A	09-11-1995	US 5543396 A	06-08-1996
WO 8903223	A	20-04-1989	US 4935493 A	19-06-1990
			AU 2801289 A	02-05-1989
			DK 658489 A	22-12-1989
			FI 900101 A	09-01-1990
WO 9308259	A	29-04-1993	CA 2121369 A	29-04-1993
			EP 0610317 A	17-08-1994
			JP 7504158 T	11-05-1995
			US 5462928 A	31-10-1995
US 5296604	A	22-03-1994	NONE	
WO 9212140	A	23-07-1992	AU 654834 B	24-11-1994
			AU 9155391 A	17-08-1992
			CA 2098702 A	29-06-1992
			EP 0564561 A	13-10-1993
			JP 6504547 T	26-05-1994
			US 5257901 A	02-11-1993